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Protection against rotavirus shedding after intranasal immunization of mice with a chimeric VP6 protein does not require intestinal IgA

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Abstract

Intranasal immunization of mice with chimeric VP6 and the adjuvant LT(R192G) consistently elicits >95% reductions in fecal rotavirus shedding following challenge. To determine the association between mucosal antibody and protection, we immunized BALB/c wt and J chain knockout (Jch−/−) mice with VP6 and either LT(R192G) or cholera toxin (CT). Both strains developed nearly equal levels of serum rotavirus IgG, but Jch−/− mice, which cannot transport dimeric IgA across epithelial cell surfaces, developed >4-fold higher levels of serum rotavirus IgA. Stool rotavirus IgA was present in wt but undetectable in Jch−/− mice. When challenged with rotavirus strain EDIM, reductions in rotavirus shedding were nearly identical in VP6-immunized wt and Jch−/− mice (i.e., 97% and 92%, respectively; $P > 0.01$). Th1 CD4 T cell responses were also detected in VP6-immunized animals based on high levels of IFN-γ and IL-2 found after in vitro VP6 stimulation of spleen cells. Therefore, protection induced by intranasal immunization of mice with VP6 and adjuvant does not depend on intestinal rotavirus IgA antibody but appears to be associated with CD4 T cells.

Keywords: Rotavirus; VP6 immunization; J chain knockout mice; Intestinal IgA

Introduction

Rotaviruses are the single most important cause of severe infantile gastroenteritis. It has been estimated that these viruses are responsible for ca. 600,000 deaths in the world annually (Parashar et al., 2003) and remain a high priority for vaccine development. To date, vaccines being evaluated in children to prevent rotavirus disease have all been live, attenuated rotaviruses administered orally. To avoid possible side effects of live vaccines (Dennehy, 2005; Glass et al., 2004), a number of non-living rotavirus vaccine candidates have been developed and are being assessed in animal models.

One such candidate is virus-like particles (VLPs). These contain the major structural proteins of rotavirus which are made using baculovirus expression systems where the expressed proteins combine to form particles that are structurally equivalent to rotavirus particles made in vivo. VLPs containing the inner capsid proteins, VP2 and VP6, either with or without the outer capsid neutralization proteins VP4 or VP7, have been used in animal models to determine their protective effects against rotavirus infection or disease. Rabbits parenterally immunized with homologous VLPs containing rotavirus proteins 2/4/6/7 were better protected against rotavirus challenge than those given 2/6 VLPs, although VP4 and VP7 were not absolutely required for protection against infection (Ciarlet et al., 1998; Crawford et al., 1999). When heterologous VLPs of any combination were used, protection decreased, and if rabbits were immunized by the intranasal route, no protection against infection was observed (Bertolotti-Ciarlet et al., 2003).

A number of groups have used the adult mouse model of rotavirus infection (Ward et al., 1990) to evaluate VLPs as vaccines. Several reports indicated that VLPs given to mice

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either parenterally or intranasally reduce fecal rotavirus shedding after murine rotavirus challenge as compared to unimmunized mice and VP7 and VP4 are not necessary to achieve protection in this shedding model (Bertolotti-Ciarlet et al., 2003; Crawford et al., 1999; Jiang et al., 1999; O'Neal et al., 1997, 1998; Siadat-Pajouh and Cai, 2001). When VLPs were given orally, protection decreased significantly in some studies and was absent in others (Bertolotti-Ciarlet et al., 2003; O'Neal et al., 1997). In a passive protection model where mouse dams were immunized, 2/6 VLPs did not prevent illness in pups, but inclusion of VP7 protected against rotavirus diarrhea (Coste et al., 2000). In the gnotobiotic pig model, VLPs administered intranasally were not protective but boosted protection from rotavirus shedding and diarrhea when administered after initial infection with an attenuated live rotavirus (Azevedo et al., 2004; Gonzalez et al., 2004; Iosef et al., 2002; Nguyen et al., 2003; Yuan and Saif, 2002; Yuan et al., 2000, 2001).

We have evaluated a new vaccine candidate based solely on one rotavirus protein, i.e., VP6, expressed in *E. coli* as a chimeric protein containing maltose binding protein (MBP). We subsequently reported that intranasal or oral immunization of mice with MBP::VP6 proteins from either a murine (EDIM) or human (CJN) strain of rotavirus protected mice of different strains and different haplotypes from shedding when challenged with either of two different stains of murine rotavirus (EDIM or EMcN) (Choi et al., 1999, 2002a). In all studies with VLPs and VP6 vaccine candidates, the levels of protection were dependent on the route of immunization, dose of immunogen and co-administration of an effective adjuvant (Bertolotti-Ciarlet et al., 2003; Choi et al., 2002b). The most effective adjuvants for mucosal immunization have been cholera toxin (CT) and *E. coli* heat labile toxin (LT) or genetically attenuated versions such as LT(R192G) (Choi et al., 2002b; O'Neal et al., 1998).

Protection in most studies with VLPs in rabbits, mice or gnotobiotic pigs correlated with levels of intestinal antibody (Gonzalez et al., 2004; Nguyen et al., 2003; O'Neal et al., 1997; Yuan and Saif, 2002), although T cell responses were not reported. The most abundant antibody at the intestinal surface is IgA but lower levels of IgM and IgG antibody producing cells can be found in the intestinal lamina propria (Mestecky et al., 2005). Dimeric IgA and pentameric IgM, but not IgG, are actively transported across the epithelial cell to the lumen of the intestine. Dimeric IgA, containing a J chain, binds to the polymeric Ig receptor (pIgR) expressed on the basolateral side of intestinal epithelial cells. After binding, the pIgR-antibody complex is transported through the epithelial cell and released from the apical surface into the lumen. During transport, the receptor is proteolytically cleaved resulting in the formation of secretory IgA (sIgA) (Kaetzel and Mostov, 2005). A genetically engineered mouse strain has been developed in which the J chain has been disrupted (Hendrickson et al., 1995). In these mice, transport of dimeric IgA by the pIgR is impaired. Although some forms of IgA can be detected in mucosal secretions, these mice lack dimeric or higher polymeric forms of IgA at mucosal surfaces which are thought to be necessary for the ability of the antibody to function (Hendrickson et al., 1996). In a study using these J chain knockout mice (Jch−/−), intranasal immunization with 2/6 VLPs and CT as adjuvant was reported to confer little protection against rotavirus shedding as compared to that found in immunologically normal mice. This study concluded that transport of non-neutralizing antibody via the pIgR-dependent pathway was necessary for protection after immunization with 2/6 VLPs (Schwartz-Cornil et al., 2002).

Mucosal immunization with MBP::VP6, either intranasal or oral, was found to elicit serum rotavirus IgG responses but small or undetectable levels of serum or stool rotavirus IgA (Choi et al., 1999). Using in vivo depletion of either CD8 or CD4 T cells in B cell-deficient mice, as well as adoptive transfer of immune cells into chronically shedding Rag-1 mice, we found that CD4 T cells were the only lymphocytes needed for protection against rotavirus shedding after intranasal immunization with MBP::VP6/LT(R192G) (McNeal et al., 2002). Subsequently, we also found that protection against EDIM shedding was retained after oral MBP::VP6/LT(R192G) immunization of B cell-deficient mice depleted of CD8 T cells but was lost when these orally immunized mice were depleted of CD4 T cells prior to challenge (unpublished results). This suggested that CD4 T cells were the only lymphocytes required for protection after either intranasal or oral immunization of mice with MBP::VP6/LT(R192G). Based on possible differences in the importance of antibody after intranasal immunization with VLPs versus chimeric VP6, coupled with evidence for large differences in the levels of protection found after oral immunization, these two immunogens may induce different protective immune responses.

In order to verify this hypothesis, the present study was conducted to determine if transport of intestinal dimeric IgA or the presence of this antibody at the luminal surface was necessary for the protection induced in mice after immunization with chimeric VP6. Normal wild-type (wt) BALB/c mice and J chain knock out mice (Jch−/−) were immunized with MBP::VP6 protein and either LT(R192G) or CT and subsequently challenged with the EDIM strain of murine rotavirus. Protection against rotavirus shedding was nearly equivalent in the two strains of mice. Immune responses were measured by determining antibody responses in wt and Jch−/− mice. Although both serum and intestinal rotavirus IgA levels were different in the two mouse strains prior to challenge as expected, memory T cell populations were induced to produce similar cytokine profiles after in vitro stimulation. Therefore, protection after intranasal immunization with MBP::VP6 did not depend on the transport by epithelial cells or the presence of dimeric IgA at the intestinal surface.

Results

Rotavirus IgA is not detectable in stools of Jch−/− mice after intranasal immunization with MBP::VP6 and adjuvants

Prior to determining the effects of the absence of J chain on protection of BALB/c mice against EDIM shedding after intranasal MBP::VP6 immunization, the levels of serum and

stool rotavirus antibodies were compared in immunized BALB/c wt and Jch−/− mice in specimens collected on the day before EDIM challenge. For this, mice were intranasally immunized with two doses of 9 μg of MBP::VP6 and either 5 μg of CT or 10 μg of LT(R192G), separated by 2-week intervals. Four weeks after the second immunization, serum and stool samples were collected for antibody measurements. Both immunized BALB/c wt and Jch−/− mice developed similar amounts of serum rotavirus IgG. These responses were not significantly different whether LT(R192G) or CT was used as adjuvant and also not significantly different when compared between wt and Jch−/− mouse strains (Table 1). Wt mice developed modest amounts of serum rotavirus IgA but Jch−/− mice contained significantly greater ($P < 0.0001$) levels, regardless of which adjuvant was used (Table 1). This result implied that Jch−/− mice have impaired hepatic and intestinal transport of dimeric IgA and confirmed reports that IgA levels in serum become elevated in these animals (Hendrickson et al., 1995, 1996).

Stool rotavirus IgA levels were low but measurable after immunization of wt BALB/c mice (Table 1). Differences in levels found when CT or LT(R192G) was used as the adjuvant were not significant ($P = 0.015$). As expected Jch−/− mice had undetectable levels of stool rotavirus IgA, and these were significantly different ($P < 0.0001$) compared to wt mice. This result confirms that Jch−/− mice cannot transport dimeric IgA to mucosal surfaces. Very little stool rotavirus IgG was detected and the levels were not significantly different between the two strains of immunized mice whether LT(R192G) or CT was used as adjuvant (Table 1).

Protection against rotavirus shedding stimulated by intranasal immunization with MBP::VP6 and adjuvant is unaffected by the absence of intestinal rotavirus IgA

To determine if intranasal immunization with MBP::VP6 and adjuvant would protect Jch−/− mice from rotavirus shedding after challenge, groups of unimmunized and immunized wt and Jch−/− mice were challenged with EDIM 4 weeks after the second immunization. Stools were collected daily from each mouse to determine shedding of rotavirus. Unimmunized wt mice shed virus starting 1 day after challenge with a peak on day 5 and then resolved the infection by day 8 (Fig. 1A). The unimmunized Jch−/− mice displayed a similar pattern of rotavirus shedding for the first 7 days where shedding peaked at days 4–5 and decreased by day 7. However, these mice were unable to fully resolve shedding for the 22 days they were

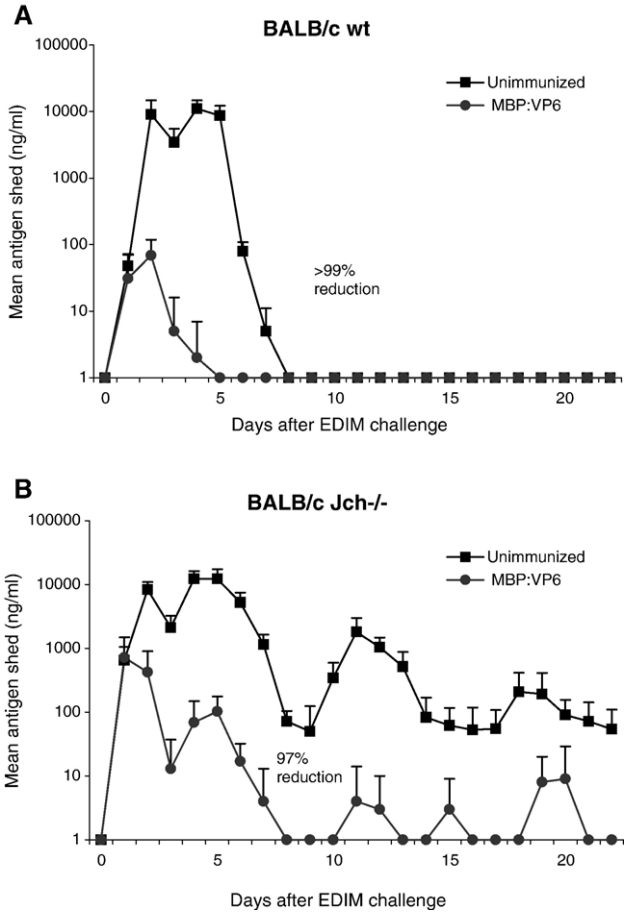

Fig. 1. Effect of intranasal immunization with MBP::VP6/LT(R192G) on rotavirus shedding in BALB/c wt (A) and Jch−/− (B) mice. Groups of adult mice were immunized with 2 doses of 9 μg MBP::VP6 and 10 μg LT(R192G). At 4 weeks after the second dose, mice were challenged with 10^5 SD_{50} of wt EDIM, and the mean shedding of rotavirus antigen (ng/mouse/day) was measured. The limit of detection for each stool specimen was 1 ng/ml. The percent reduction in shedding in the immunized mice during the first 7 days after challenge was determined relative to that found in the unimmunized mice for each strain. Standard errors of the mean are shown for each day for the mice within a group.

Table 1
Serum and stool rotavirus antibody responses after intranasal immunization with MBP::VP6[a]

Mouse strain	Immunogen	Serum IgG[b] (ng/ml)	Serum IgA[b] (ng/ml)	Stool IgG[b] (ng/ml)	Stool IgA[b] (ng/ml)
BALB/c wt	None	<100	<100	<5	<5
	MBP::VP6/LT(R192G)	299,417 ± 143,769	263 ± 143	6 ± 3	33 ± 28
	MBP::VP6/CT	341,596 ± 197,508	273 ± 201	8 ± 5	104 ± 111
BALB/c Jch−/−	None	<100	<100	<5	<5
	MBP::VP6/LT(R192G)	247,736 ± 101,066	1162[c] ± 757	6 ± 2	<5[c]
	MBP::VP6/CT	250,005 ± 95,094	1377[c] ± 622	13 ± 11	<5[c]

[a] Groups of 5 to 12 mice were intranasally immunized with two doses of 9 μg MBP::VP6 and either 10 μg of LT(R192G) or 5 μg CT. Four weeks after the second dose, serum and stool samples were obtained and the amount of rotavirus IgG or IgA was determined by ELISA.

[b] Geometric mean titers ± standard deviation. The limit of detection was 100 ng/ml and 5 ng/ml for serum and stool antibodies, respectively, and these values were used to calculate geometric mean titers for antibody levels of <100 or <5.

[c] Significantly different from BALB/c wt mice ($P < 0.0001$).

monitored (Fig. 1B). Shedding was detected in 100% of unimmunized Jch−/− on 14 of the 22 days that stool samples were collected with the lowest percentage of animals shedding on any 1 day being 70%. The level of antigen determined in the stools of individual animals after day 7 was ca. 100-fold above the limit of detection for this assay. In addition, this experiment was independently repeated two times with the same result of continued shedding. From these three experiments, 100% of a total of 26 unimmunized Jch−/− mice experienced chronic shedding.

When MBP::VP6/LT(R192G)-immunized wt and Jch−/− mice were challenged with EDIM, the amount of shedding was drastically reduced in both mouse strains when compared to the unimmunized animals of each group. Immunized wt mice shed reduced amounts of virus for 1 to 4 days, and overall, the amount of shedding was reduced by >99% during the first 7 days after challenge (Fig. 1A). Although immunized Jch−/− mice shed detectable quantities of rotavirus for more days than immunized wt mice and had additional days where low levels (<10 ng/ml) of rotavirus were detected in their stools, the amount of shedding during the 7 days after challenge was still reduced by 97% compared to that found in unimmunized Jch−/− mice (Fig. 1B). This experiment was repeated two additional times, and shedding was reduced in MBP::VP6/LT(R192G) immunized mice by 97 and 99% in wt mice and 87 and 93% in Jch−/− mice for averages of 97 and 92%, respectively.

To determine if the protection levels were different in BALB/c wt and Jch−/− mice, the percent reduction in shedding was determined for each immunized mouse of both strains as compared to the mean shedding measured for the unimmunized groups of each strain. These individual percent reductions in shedding and the mean reduction for each group are shown in Fig. 2 for each of the three experiments. The percent reductions in shedding induced in the MBP::VP6 immunized Jch−/− mice were not significantly different from the levels found in the immunized wt mice in the first two experiments when analyzed using a t test based on the Satterthwaite comparisons of unequal variances. In the third experiment, when the wt mice were compared to the Jch−/− mice using this method, differences in the levels of protection reached significance but just barely (P = 0.03). These analyses indicate that the levels of protection measured by the percent reductions in rotavirus shedding after challenge in immunized Jch−/− mice compared to unimmunized Jch−/− mice are similar to the protection levels measured in immunized wt compared to unimmunized wt mice.

When CT was used as the adjuvant during intranasal immunization with MBP::VP6, protection against shedding was also not significantly different in wt (97% reduction) and Jch−/− (91% reduction) mice. Thus, the use of either adjuvant with MBP::VP6 resulted in significant ($P < 0.0001$) protection against shedding in each strain of mice.

Similar populations of memory CD4 T cells are stimulated after intranasal immunization of BALB/c wt and Jch−/− mice with MBP::VP6/LT(R192G)

Because we have found that CD4 T cells are the only lymphocytes needed for protection after intranasal immunization of BALB/c wt and B cell-deficient (J_HD) mice with MBP::VP6 and LT(R192G), it was of interest to examine the type of memory CD4 T cell response present at the time of challenge in immunized BALB/c wt and Jch−/− mice.

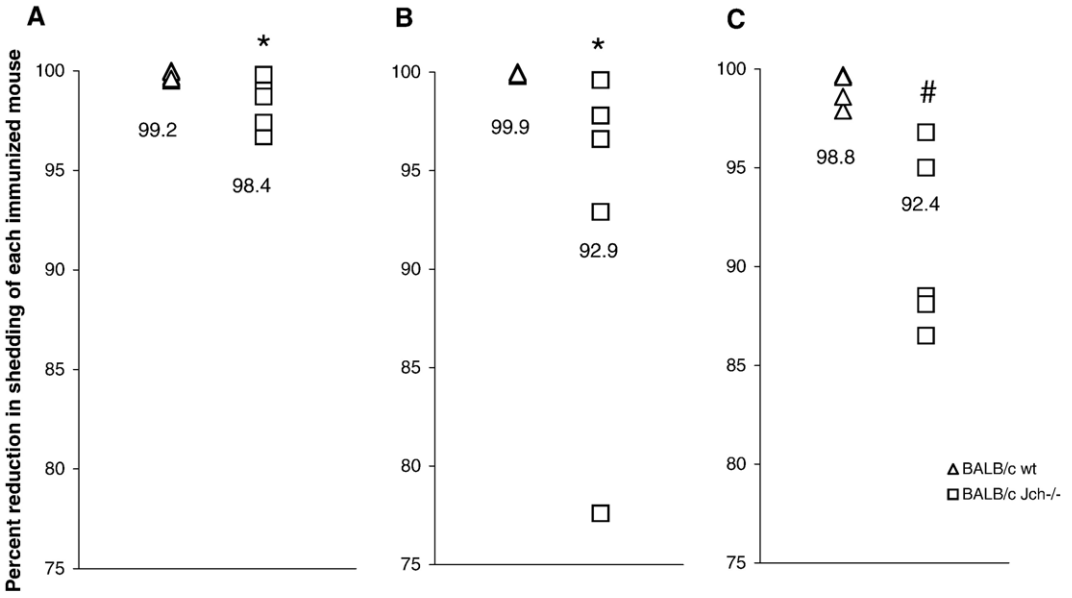

Fig. 2. Percent reductions in rotavirus shedding for MBP::VP6/LT(R192G) immunized BALB/c wt and Jch−/− mice after EDIM challenge as determined in three separate experiments (A, B, C). Groups of mice were immunized as described in Fig. 1. The percent reduction in shedding was calculated for each immunized (n = 5 or 6) mouse in a strain as compared to the mean shedding determined for all the unimmunized animals (n = 5 to 12) of each strain. Mean protection is shown for each group (wt or Jch−/−) relative to the unimmunized animals in each experiment. *Not significantly different than percent reductions of wt mice. #Significantly (P = 0.03) different than percent reductions of wt mice.

Therefore, mice belonging to both strains were immunized as already described and 4 weeks after the second immunization (the normal day of challenge), spleen cells were obtained from unimmunized and immunized animals. These cells were then either not stimulated or stimulated in vitro with MBP: :VP6. After 24 h, the last 4 in the presence of brefeldin A, supernatants were harvested and analyzed for cytokine secretion by ELISA, and the cells were stained for intracellular cytokine production. IL-2 was measured as an indicator of a recall response showing T cell recognition of the VP6 antigen. IFN-γ production was measured as an indicator of a Th1 response, and IL-4 and IL-5 were measured to indicate a Th2 response.

Unstimulated cultures produced undetectable to low levels of all cytokines measured. After in vitro stimulation with MBP: :VP6, spleen cells from unimmunized BALB/c wt and Jch−/− mice both produced little or no detectable IL-2, IL-4, IL-5 or IFN-γ as measured in the harvested supernatants (Table 2). Cells obtained from immunized animals of both mouse strains were stimulated to produce large quantities of IFN-γ and IL-2, while only undetectable to low levels of IL-4 and IL-5 were measured in these cultures (Table 2).

To determine if CD4 T cells were secreting these cytokines, the cells were stained for surface CD4 and intracellular cytokine accumulation, then analyzed by flow cytometry. No measurable cytokine production was detected in the unstimulated cells from any group (results not shown). After in vitro stimulation of spleen cells from unimmunized mice of either strain with MBP: :VP6, <0.3% of CD4 cells contained detectable IFN-γ or IL-2. However, much greater numbers of splenic CD4 cells from VP6-immunized wt and Jch−/− mice were induced to make these two cytokines (Figs. 3A and B). No measurable increases in IL-4 and IL-5 producing CD4 T cells were stimulated in immunized mice of either strain (results not shown).This result was expected since very low levels of these cytokines were measured in the supernatants. In summary, MBP: :VP6 immunization of BALB/c wt and Jch−/− mice elicited the production of rotavirus-specific, memory CD4 T cells, and in each case, these cells could be stimulated to predominantly express Th1 cytokines, thus demonstrating that CD4 T cell responses in Jch−/− mice are similar to those found in wt mice after VP6 immunization.

Discussion

Two major classes of rotavirus vaccines have been and are continuing to be developed to prevent this deadly disease. The initial candidates were all live, attenuated rotavirus strains which, after oral delivery, were anticipated to mimic the excellent protection against severe rotavirus disease elicited by natural infection. All rotavirus vaccine candidates evaluated in human trials to date have been live viruses, and two of these candidate vaccines are expected to be utilized throughout the world within the next few years. The other major class of rotavirus vaccines under development consists of either inactivated virus or subunit vaccines that can be expressed from biological vectors or delivered directly. Although many of the candidate vaccines from both classes have been found to provide excellent protection against rotavirus disease or infection in human and animal studies, the mechanisms used to elicit protection are not fully understood for any rotavirus vaccine candidate. One consistent theme of studies designed to reveal these mechanisms is to identify the memory cells responsible for protection, particularly to define the roles of CD8 and CD4 T cells as well as B cells and the antibodies they produce. The adult mouse model for studies on active immunity was designed to help define the importance of these potential effectors of protection in resolution and prevention of rotavirus infection (Ward et al., 1990).

The role of antibody in the resolution of a primary rotavirus infection has been demonstrated in a number of studies using this adult mouse model. For example, rotavirus infection in mice lacking B cells, such as μMt or J_HD strains, can result in low level chronic shedding (McNeal et al., 1995). In B cell-deficient mice, CD8 T cells are thought to be the major effectors needed to resolve or reduce rotavirus shedding because when these cells are depleted, chronic high level shedding was observed (Franco and Greenberg, 1995; McNeal et al., 1995). Prolonged shedding after primary rotavirus infection has also been observed in mice lacking functional T cells such as αβ T cell receptor knockout mice or nude mice (VanCott et al., 2001). From these studies, it was concluded that T cells were necessary for development of rotavirus antibody after infection, and

Table 2
Cytokines secreted by spleen cells obtained from unimmunized and MBP: :VP6/LT(R192G) immunized BALB/c wt and Jch−/− mice after in vitro stimulation[a]

Mouse strain	Stimulated	IL-2[b] (pg/ml)	IFN-γ[b] (pg/ml)	IL-4[b] (pg/ml)	IL-5[b] (pg/ml)
BALB/c wt unimmunized	–	18	107	55	17
	+MBP: :VP6	29	9463	64	23
BALB/c wt MBP: :VP6/LT(R192G) immunized	–	19	234	30	21
	+MBP: :VP6	3319	58,468	80	74
BALB/c Jch−/− unimmunized	–	<10	15	101	20
	+MBP: :VP6	<10	2744	85	20
BALB/c Jch−/− MBP: :VP6/LT(R192G) immunized	–	<10	801	63	13
	+MBP: :VP6	2111	145,568	163	147

[a] Groups of 2 to 6 mice were intranasally immunized with two doses of 9 μg MBP: :VP6 and 10 μg of LT(R192G). Four weeks after the second dose, spleen cells from 2–6 animals were isolated and pooled. Cells were placed in culture and either left unstimulated or stimulated with 9 ug MBP:VP6 for 24 h. Supernatants were harvested and cytokine levels determined by ELISA. Data shown is representative of two independent experiments.

[b] The limit of detection was 10 pg/ml for IL-2, IL-4, and IL-5 and 50 pg/ml for IFN-γ assays.

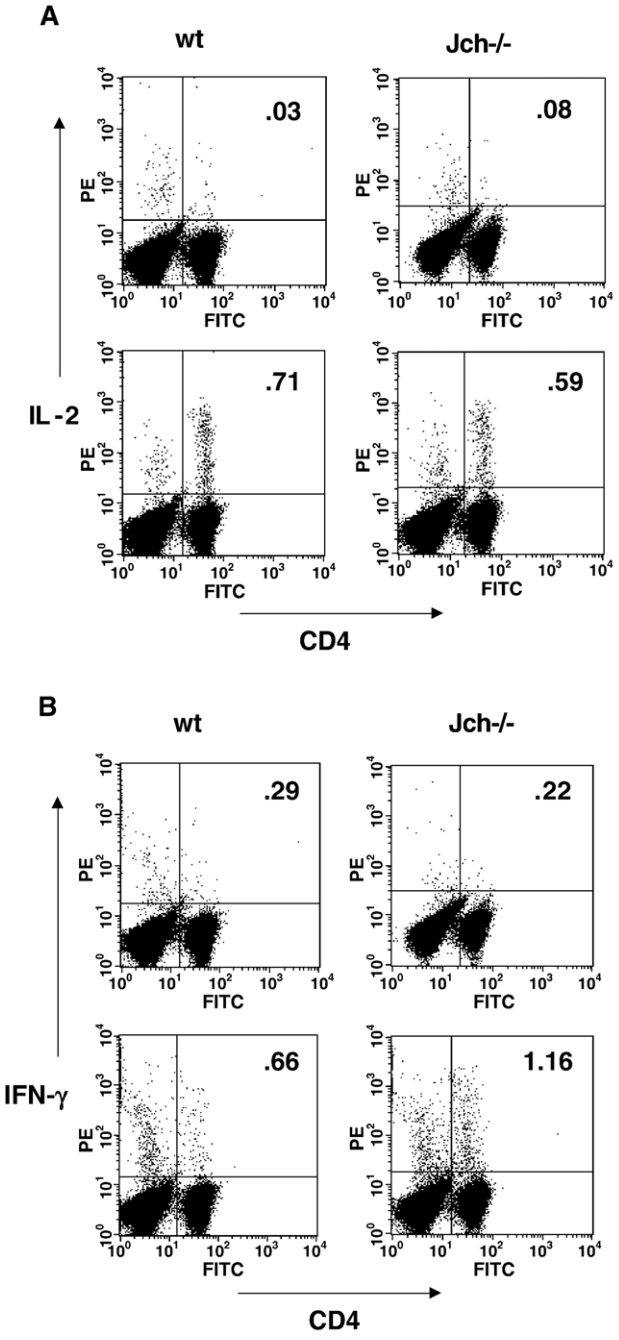

Fig. 3. Intracellular staining of spleen cells obtained from unimmunized and MBP::VP6/LT(R192G) immunized BALB/c wt and Jch−/− mice after in vitro stimulation. Cells were isolated from 2 to 6 animals and pooled. Cultures of spleen cells were stimulated with 9 μg MBP::VP6 or left unstimulated for 24 h. Brefeldin A was added for the last 4 h of culture. Cells were harvested and stained for CD4 and IFN-γ or IL-2. Cells were gated on the lymphocyte population, and the percent of CD4 cells producing cytokines was measured as shown in the upper right quadrant. Unstimulated cells had no measurable cytokine accumulation (data not shown). The top rows are cells from unimmunized mice while the bottom rows are cells from MBP::VP6 immunized animals. (A) IL-2, (B) IFN-γ. Data shown are representative of two independent experiments.

that the low levels of T-cell-independent antibody that were found in these animals were not sufficient to fully resolve rotavirus shedding.

In the present study, Jch−/− mice were used to determine the importance of intestinal rotavirus IgA after immunization with our new VP6 rotavirus vaccine candidate. These mice cannot transport dimeric IgA through epithelial cells and, therefore, lack this functional antibody at mucosal surfaces including the intestinal lumen. In a previous study conducted by Schwartz-Cornil et al. (2002), Jch−/− mice were infected with the ECw strain of rotavirus and shedding was measured after infection. The day of viral clearance was deduced from a mathematical formula derived from the viral shedding curve. The shedding curve was based on ELISA OD units instead of actual quantities of viral antigen, and the authors concluded that shedding was resolved by 8 days after infection. The authors did not collect or analyze any samples past this time point. Shedding was reduced on day 8 in their study, but in fact, their data still show measurable antigen shedding on this day. Since they did not collect additional time points, it cannot be determined if resolution really occurred in their study. In the present study, infection of unimmunized Jch−/− mice with the EDIM stain of rotavirus demonstrated a pattern of chronic shedding when monitored for over 3 weeks after the time of challenge. Rotavirus shedding did decrease by 7 days after infection but the Jch−/− mice were unable to fully resolve the infection and 100% of the animals continued to shed rotavirus at levels ca.100-fold above the limit of detection in their stools during the 22 days they were monitored. This pattern of continued shedding was similar to that found in B cell-deficient mice (J_HD and μMt mice) that lack all antibody (McNeal et al., 1995, 1997). Since Jch−/− mice have normal serum IgG levels and elevated serum IgA levels (Hendrickson et al., 1995, 1996) but cannot transport dimeric IgA to the intestinal surface, the present study demonstrates that intestinal IgA is a major effector involved in rotavirus clearance after primary infection in mice. Without intestinal dimeric IgA, CD8 T cells could not completely clear the infection. In addition, when CD8 cells were depleted at the time of challenge in Jch−/− mice, high level chronic shedding (1000- to 10,000-fold above the limit of detection) occurred in 100% of the animals and continued for over 3 weeks after infection with EDIM (results not shown). These results were similar to those obtained with B cell-deficient mice depleted of CD8 cells (Franco and Greenberg, 1995; McNeal et al., 1995).

In previous studies using IgA knockout mice, mice that cannot make any serum or stool IgA, it was found that in the absence of IgA, these mice were able to clear a rotavirus infection with a slight delay as compared to wt mice. These knockout mice were shown to have increased levels of intestinal IgM and IgG (Harriman et al., 1999). The authors concluded that these classes of antibody could compensate for the absence of IgA, perhaps due to the unusually high concentrations in this genetically modified mouse strain (Kuklin et al., 2001; O'Neal et al., 2000). Therefore, in this mouse strain (IgA knockout), intestinal antibody, although not IgA, still appeared to be the major effector for rotavirus resolution especially since shedding was resolved even when CD8 T cells were depleted in the study by Kuklin et al. (2001).

Protection against subsequent rotavirus infection induced by a previous infection, either from natural rotavirus infection or oral vaccination with live vaccine strains, has been correlated with the presence of serum or stool antibody in a number of human studies (Coulson et al., 1992; Matson et al., 1993; Ward, 1996, 2003; Ward and Bernstein, 1995). Studies in animal models using gnotobiotic pigs, rabbits, and mice have also shown correlations between serum and stool antibody produced as a result of oral infection with either homotypic or heterotypic live rotaviruses and protection against rotavirus challenge (Conner et al., 1991, 1997; Feng et al., 1997; To et al., 1998; Ward et al., 1992a, 1992b; Yuan et al., 1998). Protection against rotavirus has often been found in association with the presence of neutralizing antibody, but the importance of this antibody is unclear since protection has also been found in studies where neutralizing antibody against the challenge strain was either not detected or its levels did not correlate with protection (Ward and Bernstein, 1995; Ward et al., 1992a, 1992b, 1997).

In the present study, protection against rotavirus infection was measured after intranasal immunization with a chimeric VP6 protein and either LT(R192G) or CT using normal wt mice or a mouse strain that lacked functional intestinal dimeric IgA, Jch−/− mice. This type of vaccination represents a different route and a different immunogen when compared to a live oral immunization. It was found that even in the absence of intestinal transport or presence of dimeric IgA at the mucosal surface, these animals were protected against challenge with rotavirus. During the first 7 days following challenge, VP6 immunized Jch−/− mice shed rotavirus for more days than wt mice. In addition a low level of rotavirus antigen was detected during 5 out of the next 15 days the mice were monitored. However, immunized Jch−/− had significant reductions in shedding when compared to unimmunized Jch−/−, and the percent reductions in shedding of virus after challenge in animals immunized with VP6 were similar in wt and Jch−/− mice. This result contrasts with that reported in the study conducted by Schwartz-Cornil et al. (2002) using Jch−/− mice immunized with VLPs and CT as adjuvant. The authors of that study concluded that Jch−/− mice were not protected against challenge even though the data reported indicate a reduction in shedding in the immunized Jch−/− mice.

Several features in the two studies could explain the differences in the protection levels measured. One difference between the studies is the antigen used for immunization. The previous study used VLPs composed of bovine RF rotavirus proteins VP6 and VP2 (Schwartz-Cornil et al., 2002). The antigen used in the present study was based on one protein, i.e.,VP6 from the murine EDIM strain. It should be noted that when wt and Jch−/− mice were intranasally immunized with LT(R192G) and inactivated double-layered EDIM particles, an immunogen more similar to VLPs, shedding of rotavirus was reduced by >99% after EDIM challenge (results not shown). Although VP6 is a highly conserved protein between rotaviruses, VLPs are particles, and the VP6 within these particles may be processed and presented differently than a single protein. It is also possible that these two antigens may induce different protective responses. Protection after immunization of mice with VLPs was associated with levels of intestinal antibody (O'Neal et al., 1997, 1998). In gnotobiotic pigs, protection after VLP immunization also corresponded with the magnitude of the intestinal antibody response and when VLPs were given after an oral immunization with an attenuated live virus, they boosted the intestinal antibody responses and protection against challenge (Azevedo et al., 2004; Gonzalez et al., 2004; Iosef et al., 2002; Nguyen et al., 2003; Yuan et al., 2000, 2001). In the study by Schwartz-Cornil et al. (2002), immunization of wt mice with VLPs and CT also induced a measurable intestinal antibody response which was absent in the Jch−/− mice. Intestinal antibody lacking classical neutralizing activity has been shown to be protective against rotavirus challenge using a monoclonal antibody backpack model (Burns et al., 1996; Feng et al., 2002). Therefore, mucosal IgA induced in the wt mice after VLP immunization could be the effector of protection. However, immunization with VP6 induces little or no detectable intestinal antibody (Choi et al., 1999). In addition, we have found that B cells and CD8 T cells are not necessary for protection after VP6 immunization, and CD4 T cells were the only lymphocytes needed for protection after intranasal immunization of wt and B cell-deficient J_HD mice with VP6 (McNeal et al., 2002). The present study further confirms that antibody, specifically intestinal dimeric IgA antibody, is not necessary for protection from rotavirus challenge after intranasal immunization with VP6. However, this does not imply that intestinal antibody would not be an effector of protection if it was present; it only indicates that immunization with VP6 produces a protective immune response that is not dependent on intestinal dimeric IgA.

To date, very few studies have examined T cell responses in mice after immunization with VLPs. In a study by Fromantin et al. (1998), cytokine secretion after in vitro stimulation of lymphocytes obtained from mice immunized with VLPs with or without adjuvants was measured. The results were that after 5 days of in vitro stimulation with rotavirus, significant levels of IFN-γ, IL-4, IL-5 and IL-2 were detected by ELISA. The authors concluded that a mixed Th1/Th2 immune response was generated (Fromantin et al., 1998). Unfortunately, no direct protection studies were conducted with the same immunization procedures. In the present study, we measured the CD4 T cell responses generated by VP6 immunization of wt and Jch−/− mice by looking at cytokine expression after in vitro stimulation for 24 h. We found little to undetectable levels of IL-4 and IL-5, Th2 cytokines, after this period of stimulation. However, high levels of IL-2 and IFN-γ were detected demonstrating a predominately Th1 response. Additional cytokines may be involved in the CD4 T cell response generated after VP6 immunization but were not measured in the present study. However, the responses measured showed a similar pattern in wt and Jch−/− mice, which were nearly equally protected against rotavirus challenge. The question of how CD4 T cells are involved in the protection elicited after VP6 immunization remains to be determined.

In summary, it has been found that immunization of mice with live virus, VLPs or VP6 protein can stimulate protective responses against subsequent infection. Protection has been correlated with the presence of intestinal antibody, CD8 T cells and CD4 T cells depending on the type of immunogen, route of immunization and use of different adjuvants. The differences in the immune responses generated in animal models with different vaccine candidates may help provide insight regarding their effectiveness in humans.

Materials and methods

Virus

The murine stain of rotavirus (EDIM) used in this study was originally obtained from M. Collins (Microbiological Associates, Bethesda, MD). The pool of virus used to challenge mice after vaccination has been described previously (McNeal et al., 2002).

Mouse strains

Breeding pairs of J chain deficient (Jch−/−) mice backcrossed 10 to 12 generations into the BALB/c strain (Taconic Laboratories, Germantown, N.Y.) were obtained from the University of Chicago Children's Hospital, Chicago, Illinois. Mice were bred in our facility and used at 6 to12 weeks of age. Age-matched wt BALB/c mice were obtained from Taconic Laboratories. Mice were housed in microisolation cages and shown to be rotavirus negative before use by analyzing serum samples for the presence of rotavirus antibodies. All animal procedures were conducted in accordance with the Cincinnati Children's Hospital Research Foundation Institutional Animal Care and Use Committee.

VP6 protein used for immunization

The construction of the plasmid containing the EDIM VP6 gene, the expression of the VP6 protein in *E. coli* as a chimera with maltose binding protein (MBP) and the purification of the expressed protein, MBP: :VP6, have been described previously (Choi et al., 1999).

Immunization and challenge of mice

Mice received two intranasal immunizations, under light sedation with isoflourane (Abbot Laboratories, Chicago, IL), of chimeric MBP: :VP6 (9 μg) and adjuvant. The adjuvants used in this study were either the recombinant *E. coli* heat labile toxin LT(R192G) (10 μg) obtained from Tulane University Medical Center, New Orleans, LA or cholera toxin (CT) (5 μg) obtained from Sigma (St. Louis, MO). Immunizations were separated by 2 weeks. Four weeks after the second immunization, serum and stool samples were obtained from the mice before challenge. Groups of 5 to 12 unimmunized and groups of 5 to 6 immunized mice were challenged with 10^5 SD_{50} of wt EDIM by oral gavage.

Detection of rotavirus antigen in stool and determination of protection

To measure the quantities of rotavirus shed in fecal specimens of mice, stool samples were collected daily for up to 22 days after rotavirus challenge. Two fecal pellets were collected from each mouse and placed into 1.0 ml of Earle's balanced salt solution and stored at −20 °C until analyzed. Samples were thawed, homogenized and centrifuged (1500 × *g*, 5 min) to remove debris before being analyzed for rotavirus antigen. Quantities of rotavirus antigen shed were determined in nanograms per milliliters of stool specimen by an enzyme-linked immunosorbent assay (ELISA) using methods previously described (McNeal et al., 1999). Protection was measured as the percent reduction in shedding when comparing the mean antigen shed per mouse per day in the immunized groups to the mean shed per mouse per day in the unimmunized groups during the first 7 days following challenge.

Determination of rotavirus antibody titers

Serum and stool samples collected prior to challenge were analyzed for rotavirus IgG and IgA by ELISA and expressed in nanograms per ml as previously described (McNeal et al., 1999). Results were expressed as the geometric mean titer of a group ± standard deviation.

Measurement of T cell responses

BALB/c wt and Jch−/− mice were either unimmunized or immunized with two doses of MBP: :VP6 and LT(R192G) separated by a 2-week interval as described above. At 4 weeks after the second immunization, the time when these mice would normally be challenged with EDIM, they were sacrificed, and their spleens were removed. The spleen cells from two to six animals in each group were pooled for analysis. Single cell suspensions of spleen cells were made, and red blood cells were lysed with 8.3 mg/ml of ammonium chloride in 0.01M Tris, pH 7.4. Lymphocytes were washed and resuspended in complete RPMI medium (Gibco, Inc., Grand Island, NY) containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml Amphotericin B, 100 μM MEM non-essential amino acids and 55 μM 2-mercaptoethanol. Spleen cells (1×10^7 cells/ml) were cultured in 24-well tissue culture plates (Falcon, BD Biosciences, Bedford, MA) in complete RPMI for 24 h. Wells of cells were either left unstimulated or stimulated with MBP: :VP6 antigen (9 μg/ml) in vitro. During the last 4 h of culture, bredfeldin A (BD PharMingen, San Diego, CA) was added to the cells. Supernatants from the cultures were harvested and frozen for determination of cytokine secretion by ELISA. To measure cytokines secreted during the period of stimulation, ELISA kits for IFN-γ, IL-2, IL-4 and IL-5 were obtained from Pierce Biotechnology, Inc. (Rockford, IL) and used as instructed by the manufacturer. The levels of secreted cytokines measured were expressed as pg/ml.

To stain for intracellular cytokine production, R-Phycoerythrin (PE) conjugated antibodies for IL-4 (clone 11B11), IL-5 (clone TRGK5), INF-γ (clone XMG1.2) and IL-2 (clone JES6-5H4) and fluorescein isothiocyanate (FITC) conjugated antibody to CD4 (clone RM4-4) and isotype controls from BD PharMingen were used at concentrations recommended by the manufacturer. After in vitro stimulation, the cells were incubated with purified antibody to block Fc receptors (clone 2.4G2) and then stained with FITC-conjugated antibody to CD4. After being fixed and permeabilized according to the manufacturers instructions using a GolgiPlug kit (BD PharMingen), cells were stained with PE-conjugated antibody for either IL-2, IL-4, IL-5 or IFN-γ. Data were acquired using a FACSCaliber and analyzed by CELLQuest software (BD Bioscience, San Jose, CA). Cells were gated on the lymphocyte population by forward and side scatter determination, and 200,000 cells were acquired. Measurement of T cell responses was performed in two independent experiments.

Statistics

Comparisons in rotavirus antigen shedding within strains of mice were carried out using two sample *t* tests to determine the significance of the reduction in shedding. Differences between groups within each strain were considered significant when the probability levels (P) were <0.01. To determine the differences in protection levels between strains, the percent reduction in shedding was determined for each mouse in the immunized groups based on the mean shedding of the unimmunized groups, and then a *t* test was used to determine if the protection rates were different between strains. Unequal variances based on Satterthwaite calculations were used, and $P < 0.05$ was considered significant in comparisons between strains of mice. Challenge experiments of unimmunized and immunized mice of both strains were repeated in three independent studies.

To determine the significance of the differences of antibody levels between groups of mice, the log of the antibody titer for each mouse in a group was used for statistical comparisons. A two-factor analysis of variance based on mouse strain and adjuvant was used to analyze the data. Pair-wise comparisons were performed using Bonferroni adjustment for P values. P values <0.01 were considered significant.

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